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Identification and partial purification of serologically defined *Boophilus microplus* larval antigens by natural ectoparasite exposure☆

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Abstract

In an effort to identify life-stage specific *Boophilus microplus* proteins that elicit a humoral response in cattle, soluble proteins were extracted from 10- to 14-day-old larvae and subsequently fractionated by size-exclusion chromatography and reverse-phase high pressure liquid chromatography. Several antigens were identified by Western blotting as potentially shared with other ixodid tick species since antibodies to these proteins were present in sera of calves not previously exposed to *B. microplus*. Six putative *B. microplus*-specific antigens were identified by antibodies in the sera of calves repeatedly exposed to *B. microplus* larvae. One of the antigens, a 19.1 kDa protein, was used in the development of a diagnostic kELISA for previous exposure to *B. microplus*. The 19.1 kDa protein did not have tryptic protease activity or inhibit bovine trypsin activity, but appeared to be allergenic in that a partially pure fraction elicited immediate-type hypersensitivity responses in calves previously exposed to *B. microplus*.

Keywords: *Boophilus microplus*; Antigen; Natural exposure; Serological

1. Introduction

The southern cattle tick, *Boophilus microplus* (Say), is an economically important ectoparasite of cattle production, and cattle health. *B. microplus* vectors the causative haemoprotozoan agents of bovine babesiosis, *Babesia bovis* and *B. bigemina*. Commercial recombinant vaccines, based upon a ''concealed'' antigen, are available for the control of *B. microplus* populations (Rand et al., 1989; Rodriquez et al., 1994), and are believed to indirectly reduce transmission of pathogens (de la Fuente et al., 1995;

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Wikel, 1996). Concealed antigens are not exposed to the host by natural infestation; therefore, host immune responses elicited by vaccination with concealed antigens are not enhanced or sustained as a result of natural exposure, and booster immunization is the only means to maintain effective concentrations of antibody. Natural exposure to the tick elicits a natural immunity characterized principally by immediate-type hypersensitivity and is most effective against the larval and nymphal life-stages (Willadsen et al., 1978). Natural immunity to *B. microplus*, acquired through repeated exposure, has been described as a partial and stable immunity (Wagland, 1975). In an attempt to explain why the level of natural immunity is not more complete, Willadsen (1980) suggested that exposed tick antigens have co-evolved with the host resulting in host–parasite compatibility.

If protective immune responses elicited by natural exposure can be described or partially protective responses can be made more potent, there is an advantage for the use of exposed antigens over concealed antigens for vaccination. The vaccinated host is exposed to the vaccine antigen by natural exposure, negating or decreasing the need for booster immunization (Pruett, 2002). Tick proteins that elicit these natural immune responses are not well characterized and additional biochemical and physiological studies are clearly needed (Brossard et al., 1991). Our specific interest is in the identification and molecular characterization of life-stage specific *B. microplus* proteins that elicit a humoral immune response in cattle through natural exposure. This study reports on the identification of serologically defined antigens in a partially purified fraction of a larval *B. microplus* protein extract.

2. Materials and methods

2.1. Tick strains, tick extracts and protein determination

Boophilus ticks are maintained in quarantine at the Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX. The Munoz strain, maintained as an acaricide susceptible control strain, was used in this study for infestation and extraction of larval proteins. To prepare a soluble larval protein extract, 5 g of frozen 10–14-day-old larvae of the F_{21} generation of the Munoz strain were homogenized in a tissue grinder in 10 mM sodium phosphate buffered saline, pH 7.2 (10 ml/g of tick larvae). The homogenate was aliquoted into 50, 1 ml tubes and allowed to extract for 3 h at 4 °C with rocking. Supernatant fluid was collected by centrifugation at 14,400 × *g* for 15 min at 4 °C. The supernatant fluid was pooled and stored frozen at −20 °C. The protein concentration of the crude extract and partially pure fractions was determined with a micro-BCA technique according to the methods of the manufacturer (Pierce, Rockford, IL).

2.2. Larval infestation of cattle for antibody production

Weaned Hereford calves ($n = 4$; calf identification numbers 512, 515, 518, and 522) were purchased from the Gillespie Livestock Co. Inc., Fredericksburg, TX. The calves were in-processed at the Knipling-Bushland U.S. Livestock Insects Research Laboratory (KBUSLIRL), Kerrville, TX where they were dewormed (SafeGuard, fenbendazole paste 10%, Hoescht-Roussel Agrivet, Somerville, NJ), given Vitamins (A and D, 2 ml, Amtech, St. Joseph, MO), vaccinated with Bovi-Shield 4 (bovine rhinotracheitis, IBR, viral diarrhea, BVD types 1 and 2, parainfluenza$_3$, PI$_3$, and respiratory syncytial virus, BRSV) and blackleg (Vision 8 with SPUR Bayer, Shawnee Mission, KS), then transported to the CFTRL in Mission, TX for infestation with *B. microplus*. Stanchioned calves were infested with 500 mg of Munoz strain larvae (F_{26}) placed under a patch. Following 4 days of larval exposure, the infestation was terminated before molt to the nymphal life-stage. To terminate the infestation, calves were dipped in the organophosphate coumaphos and released to pasture. Calves were infested, using the same regimen, every 21 days for six consecutive infestations. Blood samples were obtained by jugular venipuncture prior to infestation (pre-exposure serum) and on day 21 of the initial infestation. Blood samples were also obtained the day of subsequent infestations and 7 days following each re-infestation for the remaining exposures. Serum was collected by centrifugation and stored at −20 °C. A post-exposure serum used in Western blot experiments was collected from calf 522, 21 days following the fifth larval exposure.

2.3. Partial purification of fraction 2 with open-column chromatography

Crude *B. microplus* larval proteins were concentrated to 15 ml with an Amicon ultrafiltration device (Millipore Corp., Bedford, MA) using a YM10 membrane with a 10,000 molecular weight cutoff. The concentrated proteins were loaded onto a Sephacryl S-300 (Amersham Biosciences, Piscataway, NJ) gel filtration column (78 cm × 2.6 cm) at 4 °C and eluted with 10 mM phosphate buffered saline, pH 7.2. Individual tubes (10 ml) were collected and pooled into seven fractions: fraction 1 (tubes 16–18), fraction 2 (tubes 19–30), fraction 3 (tubes 31–43), fraction 4 (tubes 44–49), fraction 5 (tubes 50–56), fraction 6 (tubes 57–64), and fraction 7 (tubes 64–75).

2.4. High-pressure liquid chromatography (HPLC) separation of fraction F2 proteins

S-300 fraction 2 proteins were further separated by reversed-phase HPLC with a Waters Xterra RP_{18}, 5 μM, 4.6 mm × 150 mm column (Waters Corp., Milford, MA). Proteins were eluted using a linear gradient from 0.1% trifluoroacetic acid (TFA), buffer A to 100% acetonitrile containing 0.1% TFA, buffer B over 67 min. The gradient was controlled by a Waters model 680 automated gradient controller (Waters Corp., Milford, MA). Fraction 2 proteins were separated into 15 peaks and concentrated to dryness with a Savant Speed Vac SC110 (Savant Instrument Inc., Farmingdale, NY). Proteins were rehydrated in 50 mM sodium phosphate buffer, pH 7.5, with and without 0.1% sodium dodecyl sulfate (SDS).

2.5. SDS-PAGE and Western blotting

Fractionated proteins were resolved on 4–20%, Tris–HCl gradient polyacrylamide gels (BioRad Ready Gel, precast gels, Hercules, CA) using a modified method of Laemmli (1970). Samples were prepared in SDS with and without 5% 2-mercaptoethanol (2-ME), depending upon experimental requirements. Gels were electrophoresed at a constant current of 20 mAmp/gel on a Mini-PROTEAN® 3 electrophoresis cell (BioRad). Resolved proteins were stained with colloidal Coomassie (GelCode Blue Stain Reagent, Pierce, Rockford, IL) according to the manufacturers instructions. Resolved and stained proteins were evaluated by integration and molecular weight determined by densitometry (LKB Ultrascan XL Laser Densitometer, and analyzed with LKB 2400 GelScan XL software, LKB Produkter AB, Bromma, Sweden).

For Western blotting, gels containing proteins resolved by SDS-PAGE (without 2-ME) were washed two times with transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3 containing 20% by volume MeOH) for 5 min each, and then transferred electrophoretically to nitrocellulose with a Mini-Trans-Blot Cell (Biorad). The blots were briefly rinsed in distilled water and blocked for 15 min with 10% goat milk in distilled water to prevent nonspecific antibody binding. All incubations of reagents were conducted at room temperature. Blots were incubated overnight with shaking in test serum diluted 1:1000, in 10 mM sodium phosphate buffer, pH 7.2, containing 1% Tween 80, 0.01% anti-foam A and 2% goat milk (serum diluent buffer). Blots were rinsed in wash buffer (10 mM sodium phosphate buffer saline, pH 7.4, containing 0.3% Tween 20) for three, 10 min cycles. Rabbit anti-bovine IgG (whole molecule, heavy + light chains, therefore, not class specific, Sigma Immuno Chemicals, St. Louis, MO) was diluted 1:4000, in serum diluent buffer and incubated with blots for 1 h with shaking. Blots were rinsed with wash buffer for three, 10 min cycles. Horseradish peroxidase-labeled goat anti-rabbit IgG (whole molecule, heavy + light chains) conjugate was diluted 1:4000, in serum diluent buffer and incubated with the blots for 1 h with shaking. Blots were rinsed in wash buffer for three, 10 min cycles. Blots were developed in substrate solution containing *o*-dianisidine (15 μg/ml) and 0.01% H_2O_2 in 10 mM Tris–HCl, pH 7.5, for 20 min with shaking. Western blots were analyzed with the Kodak Gel Logic 440 system (Eastman Kodak Co., Rochester, NY) and molecular weight determinations made with the Kodak Molecular Imaging Software (Eastman Kodak Co.).

2.6. kELISA development for anti-19.1 kDa antibody

Optimum concentrations of all reactants were determined by checkerboard titration. Microtiter plates (Costar 9017, Corning Inc., Corning, NY) were pretreated with 200 μl per well of 0.2% glutaraldehyde, covered, and incubated for 30 min at 37 °C. The plates

were rinsed with distilled water and tapped dry. Peak 10 (containing the 19.1 kDa protein) antigen (2 μg/ml) was diluted in 0.1 M sodium carbonate buffer, pH 9.5. Antigen(s) were added to wells of the glutaraldehyde treated mictotiter plates, covered and incubated at 37 °C for 3 h. The plates were rinsed three times (Ultrawash Plus, Dynatech, Chantilly, VA) with 10-s soak cycles, with ELISA wash (0.15 M NaCl, 0.5% Tween 80) and tapped dry. The plates were blocked with 10% goat milk (200 μl/well for 15 min) and rinsed as described. Test sera were diluted 1:1000, in serum diluent (10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 2% goat milk, 1% Tween 80, and 0.01% anti-foam A) and 200 μl added per well of the microtiter plates. The plates were incubated for 30 min at room temperature with shaking, and were rinsed as described above to remove unbound antibody. Rabbit anti-bovine IgG (Sigma Immuno Chemical, whole molecule, heavy + light chains) was diluted 1:2000, in serum diluent, and 200 μl was added to wells. The plates were incubated with shaking for 15 min at room temperature and rinsed as described. Horseradish peroxodase-goat anti-rabbit IgG (Sigma Immuno Chemical, whole molecule, heavy + light chains) conjugate was diluted 1:2000, in serum diluent and 200 μl was added to wells. The plates were incubated with shaking for 15 min at room temperature and rinsed as described to remove unbound conjugate. Substrate (0.2 mM ABTS, Sigma Chemical, 1.7 mM H_2O_2, 50 mM citrate, pH 4.0) was added to each well (200 μl), and the developing color reaction was monitored at 405 nm every 2 min for 30 min with an EL808 Ultra Microplate Reader (BioTek Instruments Inc., Winooski, VT). Data are presented as the slope of the reaction rate between time 0 and 16 min.

2.7. Trypsin inhibition

The chromagenic substrate N_α-benzoyl-DL-arginine-4-nitroanilide hydrochloride (L-Bapna, Sigma Aldrich, St. Louis, MO) was used to evaluate the tryptic protease activity of peak 10 proteins. The substrate L-Bapna was solubilized in DMSO and a 1.5 mM working solution was prepared in substrate buffer (40 mM Tris, pH 8.0, 50 mM $CaCl_2$). Bovine trypsin (Sigma Aldrich, St. Louis, MO) in 0.001N HCl was used (5 μg/well) as a positive control for trypsin activity. The standard assay included 5 μl bovine trypsin (1 μg/μl) plus 200 μl of substrate (L-Bapna). The reaction was monitored at 405 nm, 30 °C, in an EL808 Ultra Microplate Reader (BioTek Instruments Inc.) with readings at 30 s intervals for 8 min. Peak 10 proteins, rehydrated after concentration in 50 mM sodium phosphate buffer, pH 7.5 without SDS, were evaluated for tryptic protease activity using 10 μl (1.5 μg) protein plus 200 μl of substrate.

Evaluation of the potential for rehydrated peak 10 proteins, without SDS, to inhibit trypsin activity was investigated by preincubation of bovine trypsin (5 μg) with increasing concentrations of peak 10 (0.15 μg/μl) proteins (5, 10, 15, and 20 μl) for 20 min at 37 °C. Substrate was added (200 μl) and the reaction monitored for inhibition. Buffer controls (50 mM sodium phosphate buffer, pH 7.5) were included. Percent activity remaining after 6 min of reaction was determined by comparing the $\Delta OD_{6\ min}$ of the reaction containing peak 10 proteins with the uninhibited bovine trypsin reaction.

2.8. Skin testing

Seropositive *B. microplus* exposed calves 512 and 522 (best responders) and a control calf 583 with no known exposure to *B. microplus* were qualitatively skin tested with peak 10 proteins by intradermal (0.1 ml) injection. Peak 10 proteins, rehydrated in 50 mM sodium phosphate buffer, pH 7.5 with SDS, were prepared at a concentration of 1 μg/0.1 ml in 10 mM sodium phosphate buffered saline, pH 7.2. The buffer control injection contained the appropriate amount of SDS. The reaction site was observed at 30 min, 1, 4, 24, and 48 h post-injection. Notable skin swellings at the injection sites (peak 10 protein and control) were measured with calipers in two dimensions (*a* and *b*) and the area calculated with the formula: peak 10 protein [$\pi(ab)/4$] − control [$\pi(ab)/4$] (Gingrich, 1982) and reported in mm^2.

3. Results

3.1. Complexity of B. microplus soluble larval proteins

Crude *B. microplus* larval proteins are represented by a complex mixture of polypeptides. Most of the

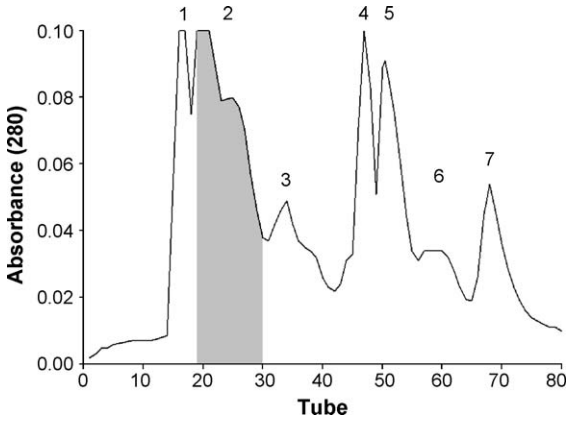

Fig. 1. Sephacryl S-300 molecular sieve chromatographic separation of soluble crude *B. microplus* larval proteins into seven fractions. Tubes combined to form fraction 2 are noted by the shaded area of the chromatogram.

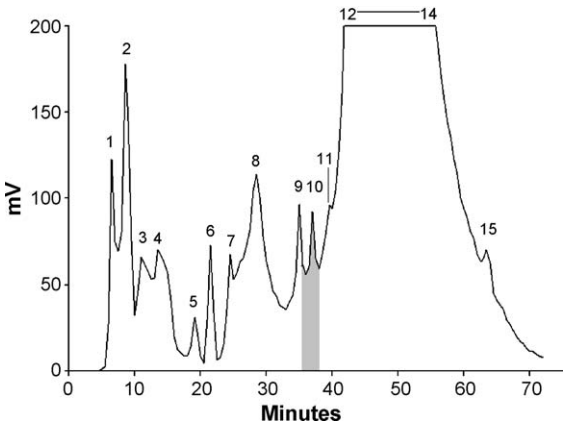

Fig. 2. Reverse-phase HPLC separation of fraction 2 into 15 peaks with a linear gradient of 0.1% TFA to 100% acetonitrile containing 0.1% TFA over 67 min. Peak 10 identified by the shaded area of the chromatogram contains the 19.1 kDa antigen.

protein bands visualized by gel electrophoresis represent a small percentage of the total protein; in terms of concentration, no single protein is dominant. Therefore, to make protein purification possible crude *B. microplus* larval proteins were initially separated on a S-300 Sephacryl size exlusion column into seven fractions to allow for the enrichment of minor proteins within specific fractions (Fig. 1). SDS-PAGE (2-ME) analysis (denatured and reduced; data not shown) of fractions 1, 2, and 3 revealed a total of 78 Coomassie stained bands with 9 bands shared between fractions. Fraction 1 contained 35 polypeptides ranging from 187.7 to 15.3 kDa, fraction 2 contained 24 polypeptides ranging from 177 to 12.9 kDa, and fraction 3 contained 32 polypeptides ranging from 94.6 to 12.3 kDa. Fraction 2, having the greater protein concentration and containing serologically defined protein antigens by initial kELISA experiments (data not shown), was made the subject of the current study (Fig. 1).

3.2. Reverse-phase HPLC separation of fraction 2 proteins

Fraction 2 proteins were further separated by reverse-phase HPLC into 15 peaks (Fig. 2, peaks 1–15). These peaks were collected, concentrated to dryness and rehydrated for SDS-PAGE (without 2-ME) analysis. Protein bands were detected with colloidal Coomassie staining in peaks 9 through 15 (Fig. 3). Peak 9 contained two distinct bands at 13.1 and 15.1 kDa, and peak 10 shared the 13.1 kDa band in addition to having a prominent band of 19.1 kDa. Peaks 9 and 10 also seemed to share minor bands between the prominent 13.1 and 19.1 kDa bands, as did peak 11. Multiple bands began to appear in peaks 12 through 14, with many of them shared. Peak 13 had the most bands with 28 determined by integration. Peak 15 contained only 2 faint bands of 86.7 and 99.2 kDa.

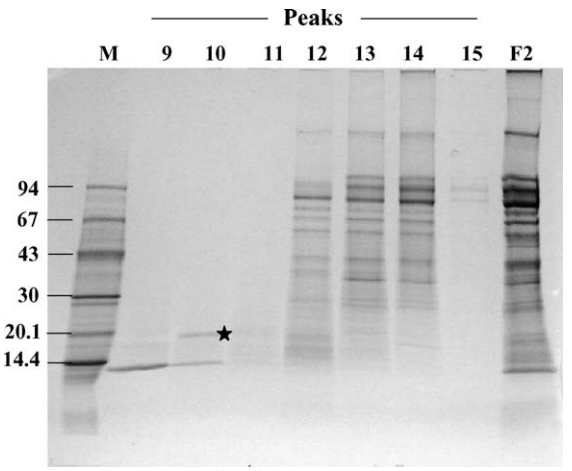

Fig. 3. SDS-PAGE (without 2-ME) analysis of fraction 2 proteins separated by RP-HPLC. Peaks 9 through 15 were resolved on a gradient (4–20%) polyacrylamide gel. M denotes molecular weight markers (kDa), and F2 the total proteins in fraction 2. The 19.1 kDa protein in peak 10 is identified by the star.

3.3. Western blot analysis of HPLC fractionated fraction 2 proteins

The proteins in peaks 9 through 15, as presented in Fig. 3, were blotted onto nitrocellulose membranes and probed with sera from calf 522 obtained from either pre-*B. microplus* larval exposure or post-*B. microplus* larval exposure (Fig. 4). As can be seen in Fig. 4A, serum collected prior to *B. microplus* larval exposure contained antibodies that bound to several *B. microplus* larval proteins. Peak 12 contained a single prominent band at 101.1 kDa, peak 13, 3 prominent bands at 107.6, 72.6, and 51.1 kDa, and peak 14 with prominent bands at 216, 105, 71.3, and 50.4. Peak 15 contained a single prominent band at 110.1 kDa. Peaks 12 through 15 appeared to share many of these proteins as evidenced by minor staining.

Serum from post-larval exposure was then incubated with these same proteins and yielded different results. The most notable difference was in peak 10 where the 19.1 kDa band identified in Fig. 3, was bound by antibodies in post-infestation antiserum (Fig. 4B), but did not react with pre-infestation serum. By Western blotting, this band appears to be a specific marker for *B. microplus* larval exposure as no antibody binding was noted in pre-infestation antiserum. Additional bands that may be specific for *B. microplus* exposure include 337.6 kDa (peak 12), 288.4 kDa (peaks 12, 13), 132 kDa (peak 12), 102.3 kDa (peak 15; represented in Fig. 3 as 86.7 kDa), and prominent band 98.2 kDa (peaks 12–14). It should be emphasized that slight differences in estimated molecular weights of bands on gels and blots may not necessarily represent real differences between those bands observed.

3.4. Development of a kELISA specific for B. microplus larval exposure

Initial attempts at developing a kELISA specific for *B. microplus* larval exposure based upon fraction 2 larval proteins as plate antigens yielded poor results with signal to noise ratios of ≈1.0 (positive/background). That is, post-infestation sera could not be distinguished from pre-infestation sera because of the high background antibody binding in pre-infestation antiserum as demonstrated in Fig. 4A. Identification of

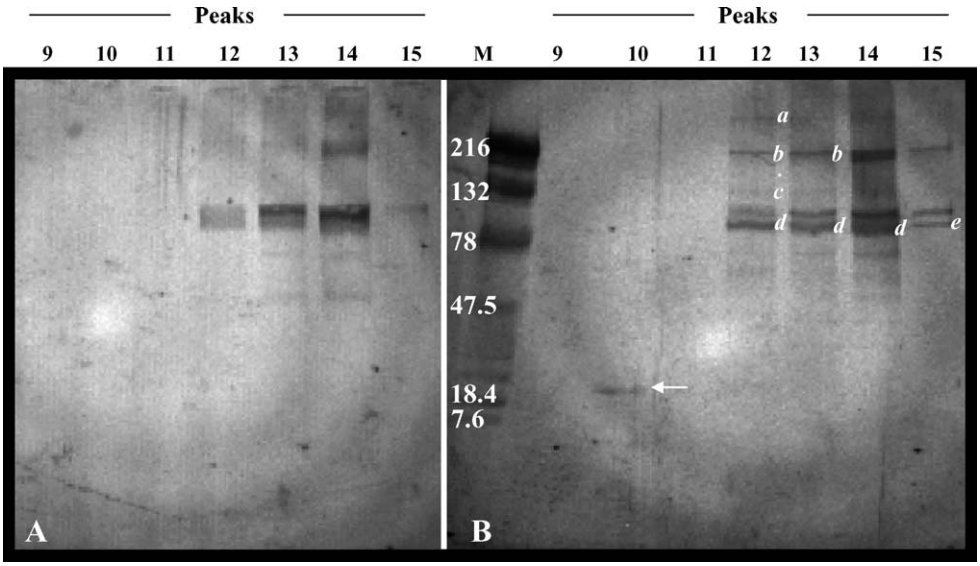

Fig. 4. Western blot analysis of fraction 2 separated HPLC peaks 9 through 15 for serologically defined protein antigens, by natural parasite exposure. Serum from calf 522 (best responder by kELISA) was used in the experiment. Blot A was exposed to serum from calf 522 prior to *B. microplus* larval exposure demonstrating potentially *B. microplus* related antigens with other ixodid tick species. Blot B was exposed to serum from calf 522 collected 21 days post the fifth exposure to larvae of *B. microplus*. Potentially *B. microplus*-specific antigens are denoted by (a) 337.6 kDa, peak 12, (b) 288.4 kDa, peaks 12 and 13, (c) 132 kDa, peak 12, (d) 98.2 kDa, peaks 12–14 and (e) 102.3 kDa, peak 15. The 19.1 kDa antigen in peak 10 is identified by an arrow. M denotes molecular weight markers (kDa). Proteins were resolved by SDS-PAGE (without 2-ME) on a 4–20% polyacrylamide gel.

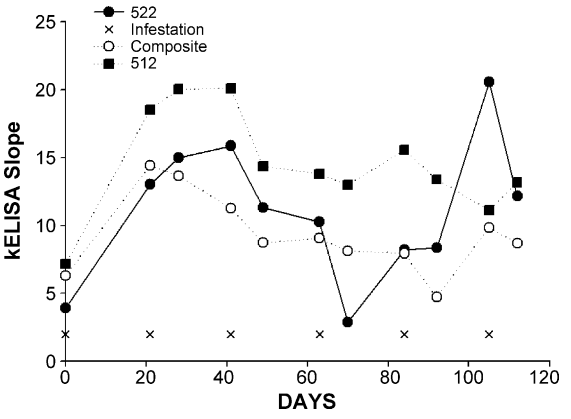

Fig. 5. Kinetic development of anti-peak 10 proteins (19.1 kDa) in calves repeatedly exposed to 4-day infestations with *B. microplus* larvae. Infestations are denoted by ×. Results of calves 522 and 512 are presented with a composite sample represented by an equal pooling of serum from all calves (512, 515, 518, and 522).

the 19.1 kDa protein, a larval protein that appears to be immunogenic in cattle (Fig. 4B) and specific for *B. microplus* larval exposure, allowed for the development of a kELISA that permitted the examination of the kinetic development of anti-19.1 kDa antibody with time and repeated exposure (Fig. 5). Calves exposed to larvae for only 4 days developed a classical primary antibody response to the 19.1 kDa protein followed by an anamnestic antibody response to the second larval exposure. Measured antibody concentrations were very erratic beginning with the third larval exposure and this pattern continued through subsequent exposures.

3.5. Peak 10 proteins were not proteolytic and did not inhibit trypsin activity

No tryptic protease activity was detected when proteins of peak 10 were evaluated for their ability to hydrolyze L-Bapna, a synthetic substrate for trypsin activity. In addition, the proteins of peak 10 did not inhibit the activity of bovine trypsin (data not shown).

3.6. Peak 10 proteins elicit immediate-type hypersensitivity responses in repeatedly exposed cattle

Calves 512 and 522, which had six exposures to *B. microplus* larvae, were qualitatively skin tested with 1 μg of HPLC peak 10 proteins (19.1 kDa) along with control calf 583 that had no known exposure to *B. microplus*. Skin testing was performed approximately 6 months following the last exposure to *B. microplus* larvae. Peak 10 proteins elicited immediate-type hypersensitivity reactions, evidenced by skin swelling, in both experimental calves, with a mean 30 min reaction of 874.7 mm^2, and a mean 1 h reaction of 1474.7 mm^2. The control calf response was 252.8 mm^2 at 30 min and 262.0 mm^2 at 1 h. The reaction sites in all calves had subsided by 4 h and neither experimental nor control calves had measurable 24 or 48 h skin reactions.

4. Discussion

Identification of expressed larval proteins of *B. microplus* that elicit a humoral immune response in the cattle host, as a result of natural exposure, is the principal purpose of the current investigation. This would most likely include those larval proteins that are exposed to the host via the salivary glands as a result of successful larval feeding. Although there is some question regarding the regurgitation of gut contents into the host during feeding (Kemp et al., 1982), the salivary gland is an important portal for host exposure to tick proteins (Ribeiro and Francischetti, 2003). Due to the complex composition of the crude larval protein extract, the experimental approach used in this study, was based upon a systematic separation of the extract into partially purified fractions, enriching lesser moieties, in order to identify those larval proteins that elicit host humoral responses by natural host–parasite interaction.

Cattle, *Bos indicus* and *B. taurus*, acquire resistance to a variety of tick species with repeated exposure (Wikel and Whelan, 1986). Host resistance is manifested primarily in the inability of life-stages to attach and obtain an adequate blood-meal, presumably in response to those antigens that the host is exposed to during attachment and subsequent feeding (Wikel, 1988; Panda et al., 1993). Inflammatory and immunological responses of hosts against ticks are documented for laboratory animals and cattle (Brossard et al., 1991); however, the antigens that induce these natural reactions are less well known, and more biochemical and physiological investigations are needed.

A number of studies have been conducted upon antigens of various tick species that were identified as immunogenic by Western blotting following vaccination with crude tick protein preparations (Wikel, 1988). However, the role of the vast majority of the Western blot-reactive moieties in the expression of host resistance remains speculative (Wikel, 1988). Wikel (1988) further suggested that the next stage of research should be the isolation, characterization and identification of their role in host acquired resistance.

Information on life-stage specific proteins of ixodid ticks that elicit host immune responses, as a result of natural exposure, is quite limited. Shapiro et al. (1986) identified 12 *Rhipicephalus appendiculatus* major antigens ranging from 120 to 16 kDa by Western blotting with serum from resistant guinea pigs. In the current study we found that serum of presumably *B. microplus* naïve calves contained antibodies that bound to *B. microplus* larval proteins (Fig. 4). This would be indicative of prior exposure to other ixodid tick species, or organisms that would elicit cross-reactive antibodies. The experimental calves were considered to be *B. microplus* naïve, as the tick has been eradicated from the U.S. for a number of years (Graham and Hourrigan, 1977), making natural exposure highly unlikely. However, exposure to other ixodid ticks, namely *Amblyomma* spp. and *Dermacentor* spp. in this region, is quite likely. These apparent cross-reacting antibodies create nonspecific background problems when developing species-specific diagnostic assays.

Additional bands recognized by pre-exposure serum, but enhanced in post-exposure sera, were also noted in peaks 12 through 15. They may represent common antigens shared between tick species, recognized by antibodies in pre-exposure serum and increased in concentration in post-exposure serum due to repeated *B. microplus* larval exposure. These bands would include the 216, 110.1, 72.6, and 51.1 kDa proteins. However, we have identified several larval proteins (337.6, 288.4, 132, 102.3, and 98.2 kDa) that may be specific to *B. microplus*. Of particular interest was a 19.1 kDa serologically defined larval protein, as it was the most obvious antigen identified as a result of *B. microplus* exposure (Fig. 4).

The fact that the 19.1 kDa protein isolated from fraction 2 in this study elicits an antibody response in calves suggests that it was likely exposed to the host via the salivary gland. An ixodid salivary protein of similar molecular weight (20 kDa) was identified by Brown et al. (1984) from *Ambloymma americanum*. The 20 kDa protein was thought to be a component of tick cement and was imunogenic in guinea pigs and elicited protective immunity in vaccine/challenge experiments (Brown and Askenase, 1986). Willadsen and Riding (1979) also isolated a protein of a similar molecular weight (18.5 kDa) from an extract of *B. microplus* larvae that was allergenic and inhibited trypsin activity. The 19.1 kDa protein identified in the current study neither exhibited tryptic protease activity nor inhibited trypsin, although it was allergenic in cattle as confirmed by immediate-type hypersensitivity responses in *B. microplus* exposed calves. Reverse-phase HPLC peak 10, the peak that contains the 19.1 kDa protein and was used in skin testing, is contaminated with other proteins (Fig. 3). However, the only protein in that fraction binding antibody in Western blots was the 19.1 kDa protein, data adding support to the allergen classification (Fig. 4). As stated, the 19.1 kDa protein was purified by reverse-phase HPLC with acetonitrile, a potential denaturant, so the absence of biological activity associated with tryptic activity and trypsin inhibition could be due to a loss of conformational integrity associated with denaturation.

Although it is tempting to speculate that the 19.1 kDa protein identified in this study and those ixodid proteins described in previous studies are the same or related, as they have similar molecular weights and they have been demonstrated to be immunogenic by natural exposure, their molecular identities await sequence information. To begin the establishment of a sequence database for serologically defined *B. microplus*-specific proteins from the various life-stages, we are currently obtaining amino acid sequence information for the 19.1 and 98.2 kDa protein antigens identified in this study.

The 19.1 kDa protein has the potential to be a diagnostic marker for *B. microplus* exposure. Calves exposed to larvae for only 4 days developed a classical primary antibody response to the 19.1 kDa protein followed by an anamnestic antibody response to the second larval exposure (Fig. 5). The erratic nature of antibody kinetics observed, beginning with the third exposure, may be explained by the feeding success of the larvae, and therefore the immunizing dose of

antigen to which the host was exposed. Development of a kELISA using peak 10, containing the 19.1 kDa antigen as plate antigen, demonstrates the potential the assay has for surveying cattle for *B. microplus* exposure. However, considerable additional work needs to be done to insure that cattle repeatedly exposed to other ixodid tick species do not produce cross-reactive antibodies to the 19.1 kDa *B. microplus* protein. The Western blot results in this study were quite specific in that pre-infestation serum did not have detectable anti-19.1 kDa antibody, while the results of the skin test were more ambiguous. Although the skin response of the control calf 583 was less than that of *B. microplus* exposed calves, it was positive relative to buffer control injection sites. The immediate-type hypersensitivity skin response observed in the control calf would suggest that the calf had been exposed to related ixodid species and that those tissue-fixed antibodies bound proteins of peak 10. Alternatively, the proteins in peak 10 could have vasoactive properties nonspecifically inducing the release of vasoactive amines from inflammatory cells. Further investigation is required to answer these important questions.

The serologically defined proteins herein represent the first to be described in a series of studies to identify *B. microplus* life-stage specific proteins that elicit antibody responses in cattle through natural exposure. These proteins may play varied roles in successful tick feeding, and along with their diagnostic potential may serve as vaccine candidates for control of the tick and the diseases vectored.

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References

Brossard, M., Rutti, B., Haug, T., 1991. Immunological relationships between host and ixodid ticks. In: Parasite Host Associations: Coexistence or Conflict, Oxford University Press, Oxford, pp. 177–200.

Brown, S.J., Shapiro, S.Z., Askenase, P.W., 1984. Characterization of tick antigens inducing host immune resistance. I. Immunization of guinea pigs with *Amblyomma americanum*-derived salivary gland extracts and identification of an important salivary gland protein antigen with guinea pig anti-tick antibodies. J. Immunol. 133 (6), 3319–3325.

Brown, S.J., Askenase, P.W., 1986. *Amblyomma americanum*: physiochemical isolation of a protein derived from the tick salivary gland that is capable of inducing immune resistance in guinea pigs. Exp. Parasitol. 62, 40–50.

de la Fuente, J., Rodriquez, M., Montero, C., Lleonart, R., 1995. Control of *Boophilus microplus* infestations in cattle vaccinated with a recombinant Bm86 antigen preparation. Evidences of control of chemical-resistant strains and *Babesia bovis* transmission. In: Seminario Internacional de Parasitologia Animal: Restencia y control en garrapatas y moscas de importancia veterinaria, Acapulco, Mexico, pp. 101–111.

Gingrich, R.E., 1982. Acquired resistance to *Hypoderma lineatum*: comparative immune response of resistant and susceptible cattle. Vet. Parasitol. 9, 233–242.

Graham, O.H., Hourrigan, J.L., 1977. Eradication programs for the arthropod parasites of livestock. J. Med. Entomol. 13, 629–658.

Kemp, D.H., Stone, B.F., Binnington, K.C., 1982. Tick attachment and feeding: role of the mouthparts, feeding apparatus, salivary gland secretions and host response. In: Obenchain, F.D., Galun, R. (Eds.), Physiology of Ticks. Pergamon Press, Oxford, UK, pp. 119–168.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680–685.

Panda, D.N., Ansarp, M.Z., Sahap, B.N., 1993. Immunization of cattle with *Boophilus microplus* adult female and larval extracts: feeding, survival and reproductive behavior of ticks on immunized cattle. Indian J. Anim. Sci. 63 (2), 123–127.

Pruett, J.H., 2002. Immunological intervention for the control of ectoparasites of livestock—a review. J. Vet. Parasitol. 16 (1), 1–10.

Rand, K.N., Moore, T., Sriskantha, A., Spring, K., Tellam, R., Willadsen, P., Cobon, G.S., 1989. Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. Proc. Natl. Acad. Sci. U.S.A. 86, 9657–9661.

Ribeiro, J.M.C., Francischetti, I.M.B., 2003. Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. Ann. Rev. Entomol. 48, 73–88.

Rodriquez, M., Rubiera, R., Penichet, M., Montesinos, R., Cremata, J., Falcon, V., Sanchez, G., Bringas, R., Cordoves, C., Valdes, M., 1994. High level expression of the *B. microplus* Bm86 antigen in the yeast *P. pastoris* forming highly immunogenic particles for cattle. J. Biotechnol. 33, 135–146.

Shapiro, S.Z., Voigt, W.P., Fujisaki, K., 1986. Tick antigens recognized by serum from a guinea pig resistant to infestation with the tick *Rhipicephalus appendiculatus*. J. Parasitol. 72 (3), 454–463.

Wagland, B.M., 1975. Host resistance to cattle tick (*Boophilus microplus*) in Brahman (*Bos indicus*) cattle. I. Responses of previously unexposed cattle to four infestations with 20,000 larvae. Aust. J. Agric. Res. 26, 1073–1080.

Wikel, S.K., Whelan, A.C., 1986. Ixodid–host immune interaction. Identification and characterization of relevant antigens and tick-induced host immunosuppression. Vet. Parasitol. 20, 149–174.

Wikel, S.K., 1988. Immunological control of hematophagous arthropod vectors: utilization of novel antigens. Vet. Parasitol. 29, 235–264.

Wikel, S.K., 1996. The Immunology of Host–Ectoparasite Arthropod Relationships. CAB International, Wallingford.

Willadsen, P., McKenna, R.V., Riding, G.A., 1978. Responses of cattle to allergens from *Boophilus microplus*. Int. J. Parasitol. 8, 89–95.

Willadsen, P., Riding, G.A., 1979. Characterization of a proteolytic-enzyme inhibitor with allergenic activity. Multiple functions of a parasite-derived protein. Biochem. J. 177 (1), 41–47.

Willadsen, P., 1980. Immunity to ticks. Adv. Parasitol. 18, 293–313.